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Composition of Urinary Coproporphyrin Isomers I–IV in Human Porphyrias[1])

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Dedicated to Professor Claude Rimington in honour of his 90th birthday

Summary: The urinary distribution and relative proportions of the four coproporphyrin isomers I–IV were investigated in 50 patients suffering from hepatic and erythropoietic types of hereditary porphyrias. A highly efficient sample preparation method was applied to isolate urinary coproporphyrins, the isomer ratios of which were quantitated by isocratic ion-pair high-performance liquid chromatography. Results showed a significant decrease ($p < 0.001$) of the proportion of coproporphyrin I in acute hepatic porphyria (acute intermittent porphyria, hereditary coproporphyria, variegate porphyria, porphobilinogen synthase deficiency porphyria) as compared with chronic hepatic porphyria (porphyria cutanea tarda, chronic hepatic porphyria type B and C) (13.2 ± 5.3%, $\bar{x}$ ± S.D., vs. 31.4 ± 11.5%). Conversely, the proportion of isomer III was significantly higher ($p < 0.001$) in acute hepatic porphyria than in chronic hepatic porphyria (80.9 ± 5.2% vs. 62.2 ± 10.9%). As expected, the highest level of coproporphyrin I (90.0 ± 1.9%) was found in congenital erythropoietic porphyria.

The atypical coproporphyrins II and IV were detected in all types of porphyria analysed and ranged from 0.2 to 9.0%; no significant differences were seen between acute and chronic hepatic porphyrias. The diagnostic importance of the isomer ratios of coproporphyrins I and III has been confirmed in our study, while the significance of the atypical coproporphyrin isomers II and IV is still unclear at present.

Introduction

Porphyrias are caused by deficiencies of haem biosynthetic enzymes (fig. 1). Characteristic excretion patterns of porphyrins and porphyrin precursors – reflecting the respective enzymatic defect – are observed in these conditions.

Differential diagnosis of porphyrias requires analysis of haem precursors in urine, feces, blood and tissues of affected patients (1, 2). Complementary studies of the haem biosynthetic enzymes in blood cells help to identify the nature of the hereditary defect (1, 2). Additional diagnostic information can be obtained for some porphyrias when the ratios of the naturally occurring isomers of series I and III are determined (3). Alterations of the normal ratios of coproporphyrin isomers I and III are found in cases of both hereditary and toxic porphyrias, e. g. lead poisoning (3–5). For instance, the well-known overproduction of type I isomers in congenital erythropoietic porphyria (4, 6) or the preponderance of heptacarboxyporphyrin III in porphyria cutanea tarda (3) greatly facilitates the biochemical diagnosis of these porphyrias. On the other hand, secondary copropor-

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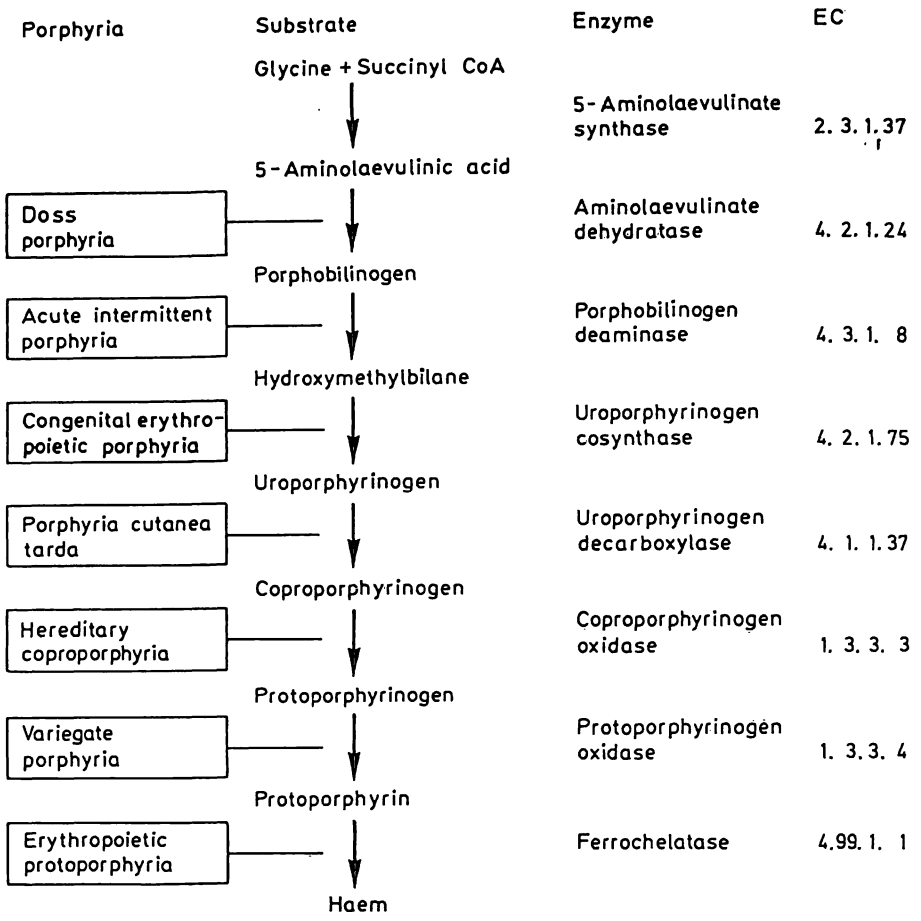

Fig. 1. Inherited enzyme blocks in the porphyrias. (Slightly modified from l.c. (28)).

phyrinurias often show characteristic isomer ratios which may originate from lead intoxication (5), some liver diseases, like alcohol- and protoporphyrin-induced cholestasis (7, 8) or hereditary hyperbilirubinaemias such as *Rotor*'s syndrome and *Dubin-Johnson* syndrome (9). Thus, the respective isomer ratios are valuable tools for the differential diagnosis of these disorders (10).

The development of enhanced high-performance liquid chromatographic (HPLC) methods enabled the simultaneous resolution of the naturally occurring type I and type III isomers in the series from uro- to coproporphyrin in a single chromatographic run (11, 12). In addition, complete resolution of the four coproporphyrin isomers I—IV was achieved under isocratic conditions (12, 13). However, the atypical isomers of the series II and IV were previously thought to be non-existent in biological materials (14). Recently, we were able to detect small amounts of coproporphyrin II and IV in human urine (15) and feces (16). These symmetrically substituted isomers are mainly formed by non-enzymatic isomerization of porphyrinogens in relatively acidic urines (17).

In this study, we investigated for the first time the isomeric composition of coproporphyrins I—IV in urine of patients suffering from the common types of hereditary porphyria. The individual isomer ratios were determined in order to clarify their potential in the differential diagnosis of porphyrias.

Materials and Methods

Patients

We analysed the isomer ratios of coproporphyrins I—IV in urine of patients suffering from acute intermittent porphyria (N = 20), hereditary coproporphyria (N = 6), variegate porphyria (N = 3), porphobilinogen synthase (synonym: δ-aminolaevulinic acid dehydratase) deficiency porphyria (N = 1), porphyria cutanea tarda (N = 10), chronic hepatic porphyria (type B and C, N = 5), congenital erythropoietic porphyria (N = 3), and protoporphyria (N = 2). Diagnosis of the respective porphyrias was based on the characteristic biochemical constellations (1—3, 8) in connection with the clinical symptoms in overt cases.

Table 1 shows the excretion of urinary porphyrin precursors and porphyrins of four porphyria patients. The patients with acute intermittent porphyria, porphobilinogen synthase deficiency porphyria and congenital erythropoietic porphyria were in a non-acute phase at the time of investigation, whereas the patient with porphyria cutanea tarda was in a clinically manifest phase. Due to the homozygous status of the patients with porphobilinogen synthase deficiency porphyria and congenital erythropoietic porphyria, their metabolite excretion is excessively increased (tab. 1).

Tab. 1. Urinary porphyrin precursors and porphyrins in four patients with various types of porphyria.

Porphyria	Acute intermittent porphyria, female, 38 years old, non-acute phase (fig. 3a)	Porphyria cutanea tarda, female, 43 years old, manifest phase (fig. 3b)	PBG synthase deficiency porphyria, male, 33 years old, non-acute phase (fig. 3c)	Congenital erythropoietic porphyria, male, 26 years old, non-acute phase (fig. 3d)	Controls, upper reference limit*
Porphyrin precursors (μmol/24 h)					
δ-Aminolaevulinic acid	175	40	1018	21	49
Porphobilinogen	315	3	8	2	8
Porphyrins (nmol/24 h)					
Uroporphyrin	950	2180	49	4918	29
Heptacarboxylic porphyrin	45	1290	25	543	4
Hexacarboxylic porphyrin	33	24	11	279	3
Pentacarboxylic porphyrin	70	265	122	1256	6
Coproporphyrin	285	205	2834	6878	119

* According to *Doss* (29)

Urines

24 h-urine samples were collected in dark brown bottles and kept frozen until analysis.

Sample preparation

Urinary total coproporphyrins were isolated by the method described previously (18) with the following modification: the TLC separation of the individual porphyrin methyl esters was performed on Kieselgel 60 (Merck, Darmstadt, Germany) using *n*-hexane/dichloromethane/acetone (41 + 41 + 18, by vol.) as the solvent (19).

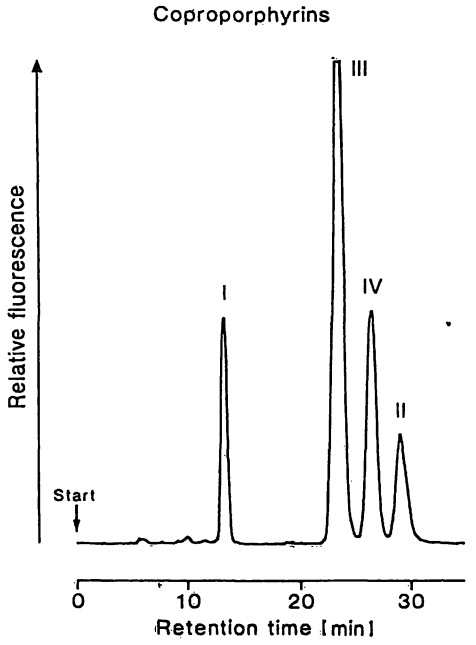

Fig. 2. Isocratic ion-pair HPLC separation of a synthetic mixture of coproporphyrin isomers I–IV containing 14.1% of type I, 52.1% of type III, 22.4% of type IV and 11.4% of type II.

Briefly, urine samples (5 to 10 ml) were oxidized with iodine, and porphyrins were then adsorbed on talc, esterified with methanol/conc. sulphuric acid, and the porphyrin methyl esters separated by TLC (see above). After hydrolysis, the free coproporphyrins were further purified by adsorption on Sep-Pak C_{18} cartridges (Waters, Eschborn, Germany) and eluted with methanol/acetone (1 + 1, by vol.).

High-performance liquid chromatographic analysis

Coproporphyrins I–IV were separated by isocratic ion-pair HPLC on a 5 μm-LiChrosorb RP-18 column (Merck) using tetrabutylammonium phosphate as the ion-pairing reagent (18). Percentages of coproporphyrin isomers were calculated from peak area ratios of the individual isomers.

Statistical analysis

The *Kolmogorov-Smirnov* test (20) was used to assess statistical significance between different variable classes.

Results and Discussion

High-performance liquid chromatographic separation and quantitation of coproporphyrins I–IV

Simultaneous separation of all four coproporphyrin isomers was achieved by means of isocratic ion-pair HPLC. Figure 2 shows the baseline separation of a synthetic mixture of coproporphyrins I–IV. The isomer ratios derived from the respective peak areas were determined with a coefficient of variation of 4% (N = 6).

Isolation of urinary coproporphyrins

The sampling procedure for urinary coproporphyrins was started with an oxidation step to prevent possible non-enzymatic isomerization of porphyrinogens under relatively acidic conditions (17, 21). The resulting porphyrins, however, are absolutely stable to further isomerization (21). Porphyrins were then isolated by adsorption on talc and TLC separation on silica gel of the respective methyl esters. For further purification, the free coproporphyrins were passed through a C_{18}-modified silica gel cartridge prior to HPLC analysis. This extensive sampling method was developed in order to obtain accurate chromatograms which were not obscured by interfering substances. This procedure was necessary to avoid erroneous results caused by contaminants co-eluting in the coproporphyrin region, especially in the case of urine samples from patients with chronic hepatic porphyria.

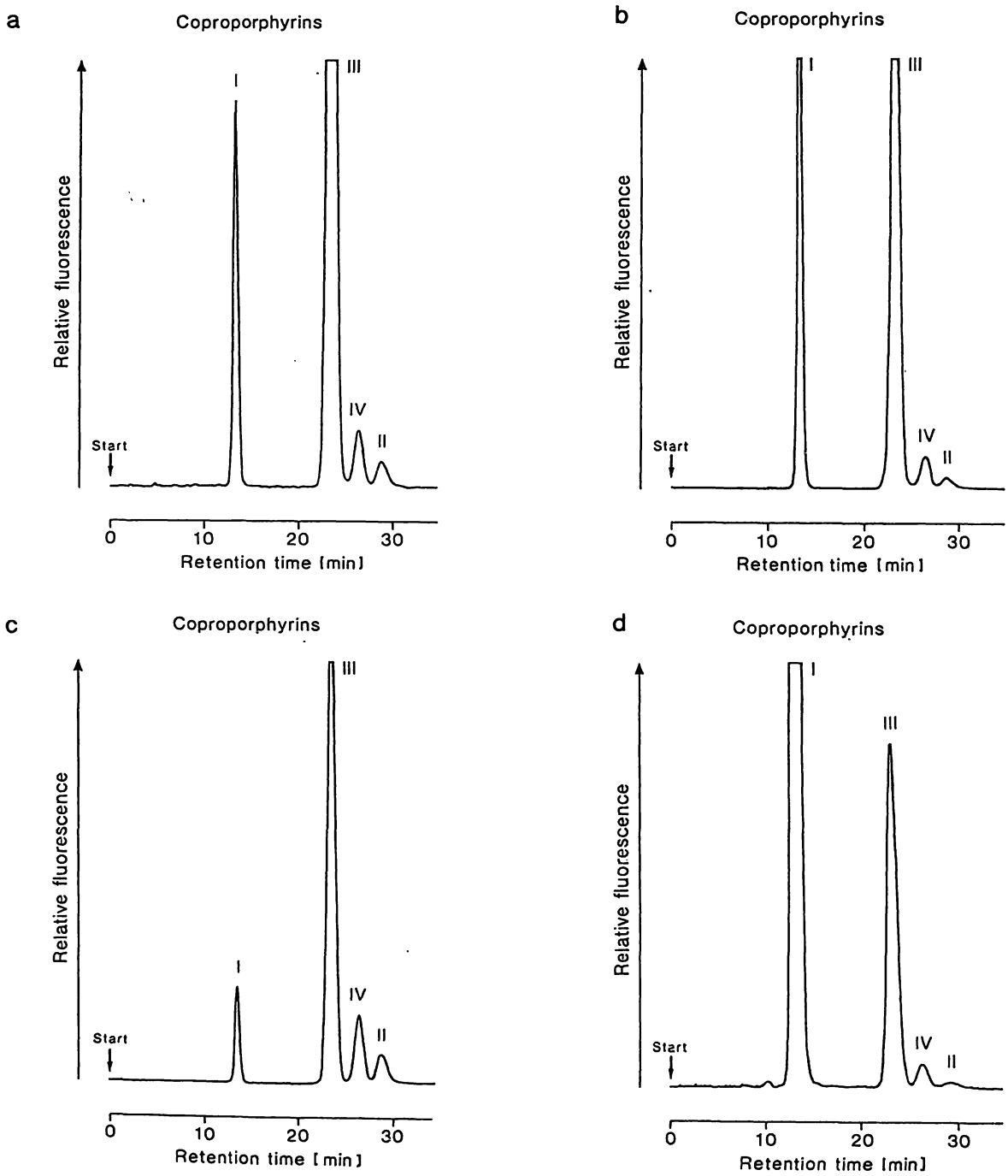

Fig. 3. Isocratic ion-pair HPLC separation of urinary coproporphyrin isomers I–IV (percentage of total coproporphyrins) from various porphyria patients.
a = acute intermittent porphyria, I (14.0), III (79.9), IV (4.1), II (2.0).
b = porphyria cutanea tarda, I (31.7), III (63.5), IV (3.3), II (1.5).
c = porphobilinogen synthase deficiency porphyria, I (5.4), III (83.4), IV (7.8), II (3.4).
d = congenital erythropoietic porphyria, I (91.9), III (7.3), IV (0.6), II (0.2).

The final yield of total coproporphyrins after extraction, esterification, TLC separation and hydrolysis varied somewhat, depending on the type of urine analysed, and was between 69 and 83%.

To check the recovery rate of the individual coproporphyrin isomers we analysed a relatively uncontaminated urine from a patient with acute intermittent porphyria by using two different methods. First, the urinary porphyrins were simply adsorbed onto a C_{18}-modified silica gel cartridge, eluted with methanol-acetone, and coproporphyrin isomers were estimated directly by isocratic ion-pair HPLC, resulting in 15.1% of type I, 79.5% of type III, 1.5% of type II and 3.9% of type IV. In a second series the same urine was analysed four times with the complete sample preparation method as described. Here we obtained 14.2 ± 1.1% ($\bar{x}$ ± S.D.) isomer I, 80.5 ± 2.4% isomer III, 1.6 ± 0.3% isomer II and 3.7 ± 0.4% isomer IV. Thus we conclude that the clean-up procedure did not alter the original isomer composition of the urine samples.

The chromatograms of urinary coproporphyrin isomers I–IV from patients with acute intermittent porphyria (fig. 3a), porphyria cutanea tarda (fig. 3b), porphobilinogen synthase deficiency porphyria (fig. 3c) and congenital erythropoietic porphyria (fig. 3d) clearly demonstrate the high efficiency of this sample preparation procedure. The clinical and biochemical data of the respective patients are presented in table 1.

Isomeric composition of coproporphyrins I–IV in porphyric urines

The isomeric composition of coproporphyrins I–IV was analysed in 50 urine specimens from porphyria patients. Mean values, standard deviations and ranges of the isomers I–IV in these samples are summarized in table 2. The isomeric composition of coproporphyrins I–IV in acute hepatic porphyrias (acute intermittent porphyria, hereditary coproporphyria, variegate porphyria, porphobilinogen synthase deficiency porphyria) versus chronic hepatic porphyrias (porphyria cutanea tarda, chronic hepatic porphyria) is compared in table 3.

In normal urines, coproporphyrins I and III account for approximately 25% and 75%, respectively, of the total porphyrins (1). In acute hepatic porphyrias, the percentage of coproporphyrin I (13.2 ± 5.3%, mean ± S.D.) was significantly lower than in chronic hepatic porphyrias (31.5 ± 11.5%, $p < 0.001$) (tab. 3). On the other hand, the proportion of isomer III was markedly higher in acute hepatic porphyrias compared with that in chronic types (80.9 ± 5.2% vs. 62.2 ± 10.9%, $p < 0.001$) (tab. 3). These findings are in agreement with results described previously by *Doss & Schermuly* (3). As expected, the highest percentage of coproporphyrin I was detected in the three cases with congenital erythropoietic porphyria (90.0 ± 1.9%). Consequently, coproporphyrin III levels were found here as low as 8.6 ± 1.2% (tab. 2). Uri-

Tab. 2. Isomeric composition of urinary coproporphyrins I–IV in various porphyrias. Data are expressed as percentage of total urinary coproporphyrins.

Porphyria	N	Coproporphyrins			
		I*	III*	II*	IV*
Acute intermittent porphyria	20	13.5 ± 5.4 (7–25)	80.7 ± 4.9 (71–89)	1.8 ± 0.9 (0.2–3.9)	4.0 ± 2.1 (0.9–8.6)
Hereditary coproporphyria	6	14.9 ± 4.3 (9–22)	79.6 ± 5.6 (75–89)	1.8 ± 1.7 (0.3–4.9)	3.7 ± 2.7 (0.6–7.1)
Variegate porphyria	3	9.5 ± 4.7 (6–15)	83.9 ± 9.4 (73–90)	1.8 ± 1.1 (1.1–3.0)	4.8 ± 3.6 (2.7–9.0)
Porphobilinogen synthase deficiency porphyria	1	5.4	83.4	3.4	7.8
Porphyria cutanea tarda	10	28.5 ± 12.0 (16–55)	64.9 ± 11.8 (39–80)	2.1 ± 1.1 (0.2–3.9)	4.5 ± 2.2 (0.5–7.9)
Chronic hepatic porphyria (type B/C)	5	37.1 ± 9.1 (24–47)	56.8 ± 6.8 (51–67)	1.7 ± 1.0 (0.5–2.7)	4.4 ± 2.0 (1.1–6.5)
Congenital erythropoietic porphyria	3	90.0 ± 1.9 (88–92)	8.6 ± 1.2 (7.3–9.8)	0.4 ± 0.2 (0.2–0.5)	1.0 ± 0.4 (0.6–1.4)
Protoporphyria	2	86.0/65.6	12.0/28.9	0.5/1.5	1.5/4.0

* mean value ± S.D. (range).

Tab. 3. Comparison of the isomeric composition of urinary coproporphyrins I–IV in acute and chronic hepatic porphyria. Data are expressed as percentage of total urinary coproporphyrins.

Porphyria	N	Coproporphyrins			
		I*	III*	II*	IV*
Acute hepatic porphyria					
Acute intermittent porphyria, hereditary coproporphyria, variegate porphyria, porphobilinogen synthase deficiency porphyria	30	13.2 ± 5.3 (5–25)	80.9 ± 5.2 (71–90)	1.8 ± 1.1 (0.2–4.9)	4.1 ± 2.3 (0.6–9.0)
Chronic hepatic porphyria					
Porphyria cutanea tarda, chronic hepatic porphyria	15	31.4 ± 11.5 (16–55)	62.2 ± 10.9 (39–80)	2.0 ± 1.0 (0.2–3.9)	4.4 ± 2.1 (0.5–7.9)
		$p < 0.001$	$p < 0.001$	$p > 0.6$ (N.S.)	$p > 0.3$ (N.S.)

* mean value ± S.D. (range).

nary coproporphyrin isomer ratios of patients suffering from protoporphyria are influenced by the degree of impaired liver function (8). Isomer I increases in cases with protoporphyrin-induced cholestatic liver disease (8). The atypical coproporphyrin isomers II and IV were detectable in all the types of porphyria analysed. They ranged from 0.2 to 9.0% of total coproporphyrins (tab. 2). Normal urines contain ca. 2% of isomer II and ca. 4% of isomer IV (15). The highest level of coproporphyrin II (4.9%) was found in the urine of a patient with hereditary coproporphyria, while the highest percentage of coproporphyrin IV (9.0%) was observed in a case with variegate porphyria. Isomers II and IV were rather low in urine of patients with congenital erythropoietic porphyria and protoporphyria (tab. 2).

In contrast to isomers I and III, there were no significant differences between the levels of isomer II (1.8% vs. 2.0%, $p > 0.6$) and isomer IV (4.1% vs. 4.4%, $p > 0.3$) in acute and chronic hepatic porphyrias (tab. 3).

Formation of the atypical coproporphyrins II and IV

Previously, we showed a marked increase of coproporphyrins II and IV in acidic urines sampled from healthy controls, due to the high content of porphyrinogens in non-porphyric urines (17). In urine samples of porphyric patients, however, the concentrations of porphyrinogens are significantly lower (22), and therefore the pH-dependent formation of the isomers II and IV is markedly reduced.

With the exception of congenital erythropoietic porphyria and protoporphyria, we found comparable percentages of coproporphyrins II and IV in porphyric and non-porphyric urines. In addition, a constant isomer ratio of approximately 1 : 2 was observed for coproporphyrins II and IV in acute and chronic hepatic porphyrias (tab. 1). Such a ratio indicates the non-enzymatic formation of these isomers, because the same ratio is found in the so-called statistical mixture of isomers I–IV, when porphyrinogens are isomerized under thermodynamically controlled conditions (21).

Diagnostic value of coproporphyrin isomer ratios

Study of the urinary coproporphyrin isomers of patients with acute and chronic hepatic porphyrias revealed highly significant differences in the distribution of isomers I and III. These indices may therefore contribute to the differentiation between acute and chronic hepatic porphyrias. In contrast to the isomers I and III, the atypical isomers II and IV are not suitable for the differential diagnosis of these porphyrias.

The high proportion of coproporphyrin I in urine of patients with congenital erythropoietic porphyria is a diagnostically important indicator for the underlying enzymatic defect, although comparably high levels can be observed in patients with *Dubin-Johnson* syndrome (10). Therefore, additional studies on the isomer ratios in cases of secondary coproporphyrinurias might be helpful for the differential diagnosis of such diseases.

Explanation of increased coproporphyrin excretion in porphobilinogen synthase deficiency porphyria

The high increase of coproporphyrin III in porphobilinogen synthase deficiency porphyria is explained by a compensatory counterregulatory enhancement

of porphyrinogen biosynthesis (23). Coproporphyrinogen oxidase activity is not reduced in this type of porphyria. An increased excretion of coproporphyrin III has also been observed in acute intermittent porphyria (3). However, there is no deficiency of coproporphyrinogen oxidase in acute intermittent porphyria, so that a compensatory mechanism that exaggerates the enzymatic defect has been proposed as background for this increased porphyrin biosynthesis (24). Furthermore, the metabolic changes can be imitated by oral δ-aminolaevulinic acid loading in humans, resulting in an excessive increase of urinary coproporphyrin excretion, containing about 90% of isomer III (3). These observations show clearly that coproporphyrin isomer III is the main component of the urinary porphyrins derived from excessive amounts of δ-aminolaevulinic acid of endogenous or exogenous origin.

Doss porphyria versus acute intermittent porphyria

Porphobilinogen synthase deficiency porphyria was first described 1979 by *Doss* et al. (23). This acute hepatic porphyria represents a special entity with a nearly complete enzyme deficiency, which differs from that of acute intermittent porphyria (fig. 1). Porphobilinogen synthase deficiency porphyria becomes clinically manifest with an acute porphyria syndrome (23) only in the homozygous state. In contrast to acute intermittent porphyria, heterozygotes are clinically non-affected (25). The enzyme defect could be traced over three generations (25). Immunological characterization of non-catalytic enzyme (cross reacting immunological material (CRIM)-positive) showed that a structurally modified enzyme is present (26). Recently, cloning and expression of the defective genes from the patient with porphobilinogen synthase deficiency have been reported (27). Two separate point mutations were found in each porphobilinogen synthase allele, suggesting a compound heterozygosity as the molecular basis for this rare type of acute porphyria (27).

Conclusion

In this study, we confirmed the occurrence of increased proportions of urinary coproporphyrin III in acute hepatic porphyria as compared with chronic hepatic porphyria (3). In contrast, the proportion of coproporphyrin I was found to be significantly lower in acute hepatic porphyria than observed in chronic hepatic porphyria.

The percentage of the atypical coproporphyrin isomers II and IV, which are probably formed non-enzymatically, sometimes exceeds 10% of total coproporphyrins in individual cases. This unexpected finding has not been reported previously. Additional studies are required on the biogenesis of the atypical isomers under physiological conditions. It is assumed that these isomers arise from rearrangement of the tetrapyrrolic ring after urine has been excreted by the kidney. In urine of healthy controls, we were able to demonstrate a stringent pH-dependency of coproporphyrin isomer ratios (17). The pathobiochemistry of altered porphyrin isomer ratios has yet to be elucidated by further investigations.

High proportions of isomer I either originate from an enzymatic defect, as in congenital erythropoietic porphyria (6), or they are formed non-enzymatically, as in *Dubin-Johnson* syndrome (8) and in protoporphyria (10). In the latter disturbances, membrane and transport processes play an important role in the alteration of the respective porphyrin isomer ratios.

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